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Altered primordial germ cell migration in the absence of transforming growth factor β signaling via ALK5

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Abstract

Transforming growth factor β (TGFβ) inhibits proliferation and promotes the migration of primordial germ cells (PGCs) towards explants of gonadal ridges in vitro. However, its effects in vivo are still unclear. Here, we analyzed the behavior of PGCs in embryos lacking TGFβ signaling via the type I receptor ALK5. TGFβ in vivo was neither a chemoattractant for PGCs, nor did it affect their proliferation during migration towards the gonadal ridges up to embryonic day (E)10. Unexpectedly, the absence of TGFβ signaling in fact resulted in significant facilitation of PGC migration out of the hindgut, due to the reduced deposition of collagen type I surrounding the gut of *Alk5*-deficient mutant embryos. Migratory PGCs adhere strongly to collagen; therefore, reduced collagen type I along the gut may result in reduced adhesion, facilitating migration into the dorsal mesenterium and gonadal ridges. Our results provide new evidence for the role of TGFβ signaling in migration of PGCs in vivo distinct from that described previously.

Keywords: Primordial germ cells; Mouse embryo; TGFβ; Migration; Collagen; Hindgut

Introduction

In mice, the primordial germ cells (PGCs) have an extra-gonadal origin. They are allocated during gastrulation around embryonic day (E)7.2 at the base of the allantois, where a founder population of about 45 cells can easily be identified on the basis of staining for tissue nonspecific alkaline phosphatase (AP) activity (Chiquoine, 1954; Ginsburg et al., 1990). To reach the gonadal ridges, the PGCs first occupy the definitive endoderm (Anderson et al., 2000), then migrate through the hindgut and into the mesentery as it forms and expands, and finally move laterally into the gonadal ridges at E10.5–E11.5 (Molyneaux et al., 2001).

Experiments on cultured PGCs suggested that two different mechanisms may contribute to regulate their migration: chemotaxis and a gradient of extracellular matrix (ECM) (Alvarez-Buylla and Merchant-Larios, 1986; Ffrench-Constant et al., 1991; Garcia-Castro et al., 1997; Godin and Wylie, 1991; Godin et al., 1990). Evidence for involvement of an ECM gradient in vivo was provided by Anderson et al. (1999), who showed that PGCs lacking the ECM receptor β1 integrin did not colonize the gonadal ridges efficiently. Recently, a

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specific requirement for the chemokine stromal cell-derived 1 (SDF1) and its receptor CXCR4 for proper colonization of the gonadal ridges has been demonstrated, although SDF1/CXCR4 signaling is not involved in the earlier phases of PGC migration (Ara et al., 2003; Molyneaux et al., 2003).

In vitro, transforming growth factor β1 (TGFβ1) has been shown to mimic the chemotropic effect of E10.5 gonadal ridges observed for PGCs isolated from E8.5 embryos, in a dose-dependent manner. Moreover, a neutralizing antibody against TGFβ1 blocked the chemotropic effect from the gonadal ridges, suggesting that indeed TGFβ is involved in the homing of PGCs (Godin and Wylie, 1991). However, since these in vitro experiments were all performed on STO feeder cells and there is no direct evidence that E8.5 PGCs express TGFβ receptors, it remains unclear whether the observed TGFβ effect was direct on the PGCs or indirect via the feeders. Nevertheless, even though at E8.5 the gonadal ridges have not yet formed and the PGCs have just entered the hindgut, at E10.5 the dorsal body wall does express TGFβ1 (Godin and Wylie, 1991), indicating that TGFβ1 could play a role regulating migration of PGCs towards the gonadal ridges in vivo.

TGFβ1 is the prototype of the TGFβ superfamily of secreted growth factors. This superfamily is subdivided in the TGFβ subfamily (TGFβ1–3, activin, nodal) and the bone morphogenetic protein (BMP) subfamily (BMPs, anti-Müllerian hormone, growth and differentiation factors). All members of the TGFβ superfamily signal through serine/threonine kinase transmembrane receptors, requiring both the type II and type I receptors to initiate a cellular response. Upon activation of type I receptors, receptor regulated (R-)Smads are phosphorylated (PSmad) in their C-terminal sequence and translocate to the nucleus where they modulate transcription (reviewed by Shi and Massague, 2003). TGFβs (TGFβ1–3) interact specifically with the type II receptor TβRII and the type I receptor ALK5 (and in endothelial cells, ALK1), but signal via the common TGFβ subfamily R-Smads, Smad2 and Smad3.

The purpose of the present study was to determine whether TGFβ(1–3) signaling contributed to PGC migration directly in vivo. The distribution of PGCs was therefore analyzed in *Alk5*-deficient mice. Notably, the results showed not only that *Alk5*-deficient embryos had normal numbers of PGCs until E10, but also that unexpectedly *Alk5*−/− PGCs colonized the gonadal ridges more efficiently than wild type controls. This strongly suggested that TGFβ does not function as a chemoattractant nor controls the proliferation of PGCs in vivo, in contrast to expectation. Our results however suggest that TGFβ signaling via ALK5 promotes ECM deposition, in particular collagen type I, around the gut, and thus indirectly restricts the movement of PGCs from the hindgut to the dorsal mesenterium.

Materials and methods

Mouse strains and embryo isolation

Wild type mice and Oct4ΔPE:*gfp* transgenic mice were BL6/CBA; *Tgfbr1*$^{+/-}$ (or *Alk5*$^{+/-}$) mice (Larsson et al., 2001) were BL6. Embryos were isolated in cold Dulbecco's minimal essential medium (DMEM, Invitrogen) supplemented with 7.5% fetal calf serum (FCS) and 10 mM HEPES. *Alk5* genotyping was performed as described (Larsson et al., 2001).

Immunohistochemistry on embryos

Embryos were fixed overnight (o/n) in 4% paraformaldehyde in PBS (PFA) and embedded in paraffin using standard procedures. Sections (6 μm) were dewaxed, rehydrated, and treated for 15 min at room temperature (RT) in 5% hydrogen peroxide solution. Sections to be stained with rabbit anti-PSmad2 (Persson et al., 1998) and anti-UTF1 (Eggen et al., unpublished) antibodies were boiled for 20 min in 10 mM sodium citrate pH 6.0, whereas sections to be stained with rabbit anti-PSmad1/5/8 antibody (Cell Signalling Technology) were boiled 20 min in 10 mM Tris/1 mM EDTA pH 9.0. Sections were blocked for 30 min at RT in 0.05% BSA/PBS, treated with the first antibody (1:50, 1:100, and 1:200, respectively) in 0.05% BSA/PBS o/n at 4°C. PowerVision™ Poly-HRP-Conjugate (ImmunoVision Technologies) was used as secondary antibody with Fast 3,3′-diaminobenzidine tablet set (DAB, Sigma). Paraffin sections to be stained with rabbit anti-fibronectin (Sigma) or rabbit anti-collagen type I (Rockland Immunochemicals) were treated as described (Soto-Suazo et al., 2004; Zwijsen et al., 1999). Cy3-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research, 1:250) was used as secondary antibody.

PGC staining and analysis

E7.5–E10.0 embryos were fixed for 2 h in 4% PFA at 4°C, dissected further to expose the area containing the PGCs and stained as whole-mount for AP activity (Lawson et al., 1999). The PGCs were counted and the distance between the most rostral and most caudal PGCs was measured. Regression analysis and comparison of regression lines were performed as described, using F to compare variances (Snedecor and Cochram, 1967). E10.5 embryos were embedded in Paraplast (Sigma) after fixation (as above) using standard procedures and stained for AP activity using ASMX/Fast Red TR (Sigma) following manufacturer's instructions. PGCs were counted in each embryo section and the Wilcoxon nonparametric rank test was used for statistical analysis (Snedecor and Cochram, 1967). In all cases, AP staining was performed maximally 3 days after embryo collection.

E9.5 hindgut/mesenterium: culture, adenovirus infection, Western blotting, and immunofluorescence

The posterior part of E9.5 embryos containing the hindgut and associated mesenterium were isolated and treated as follows (1) incubated in 1 mg/ml collagenase (Maltsev et al., 1994) for 30 min at 37°C and dissociated. Cells were resuspended in DMEM with 15% FCS and plated (1×10^6 cells/well) on gelatin-coated 4-well culture plates (NUNC). After 3 h, cells were infected with 80 moi adenovirus expressing *LacZ*, dominant-negative *Alk5* or constitutively active *Alk5* (Fujii et al., 1999), cultured for 24 h at 37°C and 5% CO_2 and collected for protein isolation and Western blotting (Faure et al., 2000). When used, TGFβ1 (Peprotech) was added to the culture medium at 10 ng/ml. (2) fixed in 4% PFA o/n at 4°C, cryoprotected in 15% sucrose/PBS o/n at 4°C, embedded in OCT, cryosectioned (10 μm) and immunostained for PSmad2. (3) treated with Trypsin/EGTA for 10 min at 37°C, dissociated and plated on coverslips coated with 0.1% poly-L-lysine (Sigma), fixed 30 min in 2% PFA at RT and immunostained with rabbit anti-ALK5 (V22, Santa Cruz) or rabbit anti-TβRII (C16, Santa Cruz) and mouse anti-Oct4 (BD Biosciences) diluted 1:100 as described (Chuva de Sousa Lopes et al., 2004). Alexa Fluor® 568 goat anti-rabbit and Alexa Fluor® 488 goat anti-mouse IgG antibodies (Molecular Probes, 1:500) were used as secondary antibodies.

E9.5 hindgut: culture and RT-PCR

The posterior part of E9.5 embryos containing the whole hindgut and associated mesenterium were isolated, incubated 10 min at RT in HBBS (Gibco) containing 2 mg/ml dispase (Gibco) and 1 mg/ml collagenase type I (Sigma). The hindgut was then mechanically separated from the dorsal mesenterium using tungsten needles. cDNA was isolated from individual hindguts and dorsal mesenteria using the kit Cells-to-cDNA™ II (Ambion) following manufacturer's instructions. PCR conditions were 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C and 45 s at 72°C, followed by 5 min at 72°C. PCR primers for Shh were 5′-TTGTAACCGCCACTTTGTCA-3′ and 5′-CGCTGCT AGGTGCACTTTTA-3′, for Bmp4 5′-GCCATACCTTGACCCGCAGAAG-3′ and 5′-AAA TGGCACTCAGTTCAGTGGG-3′, for Alk5 5′-TCCACCAGGTTTGCAATTTT-3′ and 5′-ATCTGAAAGGGCAGCAATCA-3′, for TβRII 5′-CCCTCTAGCGGGGAATTTAC-3′ and 5′-CAGCGATGCTATTCCTTGGT-3′. Primers for collagen type I and fibronectin have been described (Bohnsack et al., 2004; Maes et al., 2002).

Isolated whole hindguts were cultured in DMEM with 15% FCS for 20 h at 37°C and 5% CO_2 on coverslips coated with collagen type I (2, 20, or 200 μg/ml in 0.02 N acetic acid, BD Biosciences) or on a monolayer of mitomycin-C-treated STO feeder cells and treated with TGFβ1 (0, 5, or 10 ng/ml) fixed 2 h in 4% PFA at 4°C. Some explants were stained with DAPI.

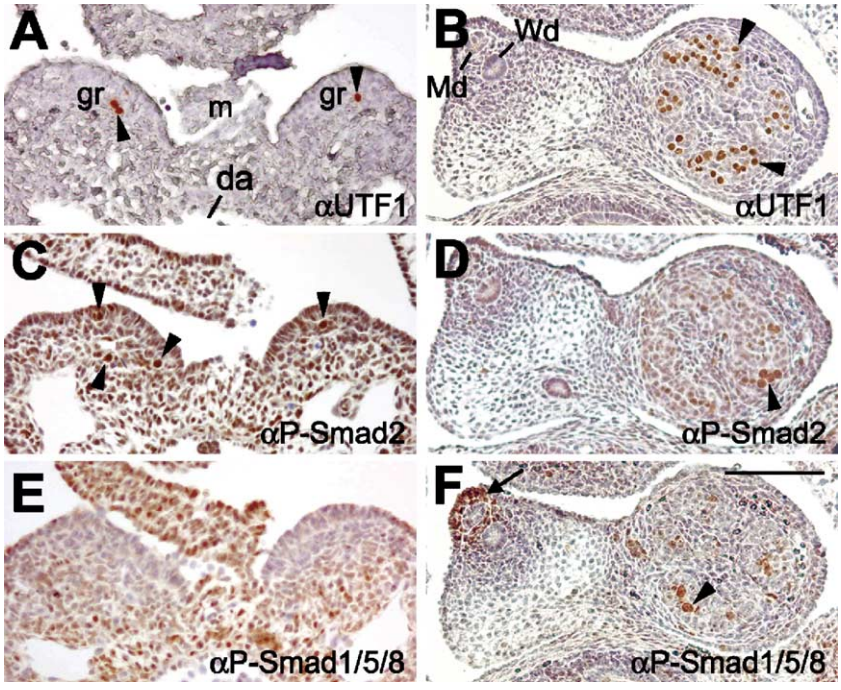

Fig. 1. TGFβ signaling during migration of PGCs at E10.5 and E12.5. (A–F) Transverse paraffin sections of E10.5 (A,C,E) and E12.5 (B,D,F) mouse embryos immunostained for UTF1 (A,B), PSmad2 (C,D), and PSmad1/5/8 (E,F), respectively. Arrowheads indicate PGCs, arrow indicates strong PSmad1/5/8 staining surrounding the Müllerian duct. Abbreviations: da, dorsal aorta; gr, gonadal ridges; m, mesenterium; Md, Müllerian duct; Wd, Wolffian duct. Scale bar: 100 μm.

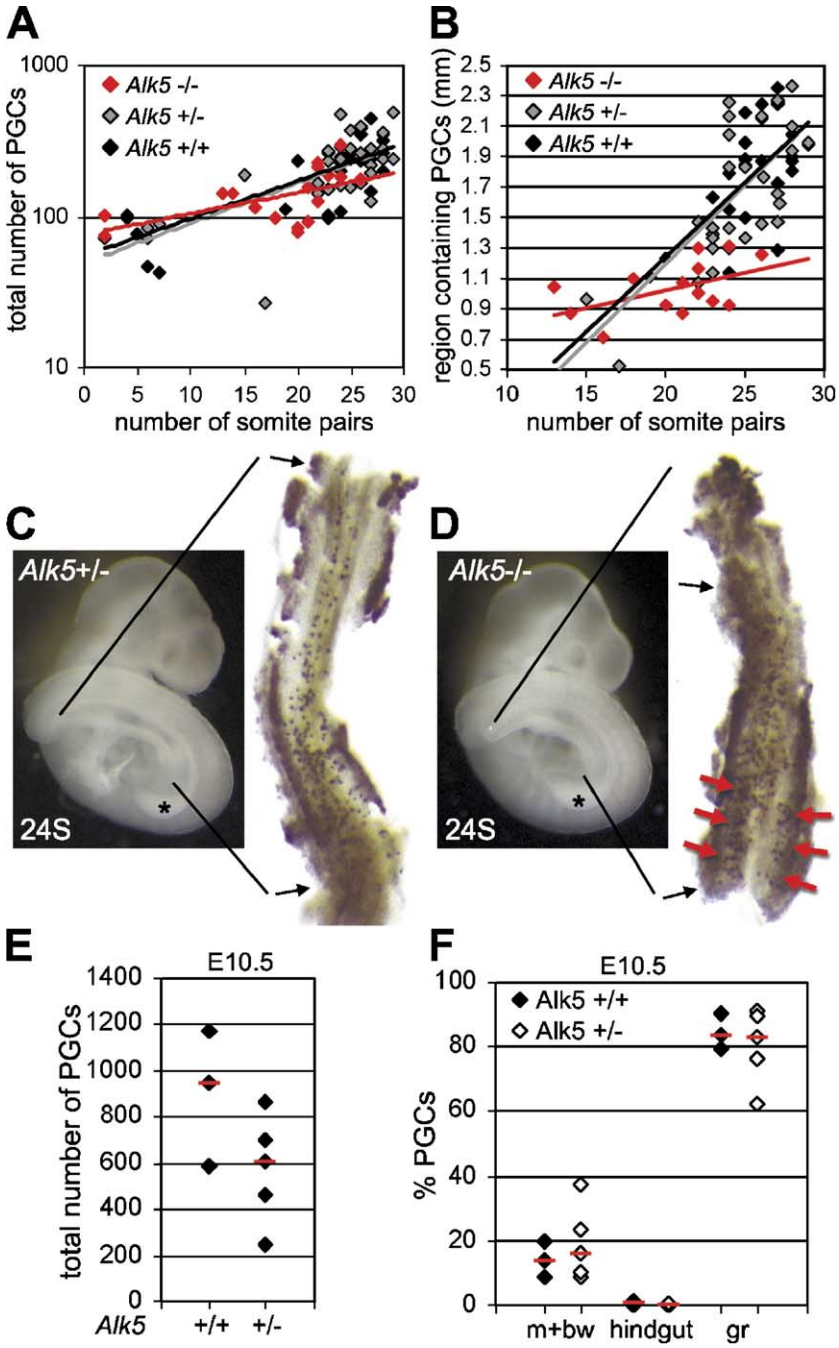

Fig. 2. PGC distribution in *Alk5*-deficient embryos. (A) Linear regression analysis of the total number of PGCs counted as whole-mounts versus the number of somite pairs, in embryos from *Alk5*+/− intercrosses. The regression equations are expressed as $y = a + bx$, for log PGC number (y) on somite number (x) at the mean values of x and y: wild type 2.262 = 1.692 + 0.0264(21.6); heterozygote 2.280 = 1.741 + 0.0247(21.8); homozygote 2.134 = 1.883 + 0.0138(18.2). (B) Linear regression analysis of the posterior (hindgut) region in length containing PGCs, from the most anterior to the most posterior versus the number of somite pairs, in embryos from *Alk5*+/− intercrosses. The regression equations are expressed as $y = a + bx$, for posterior (hindgut) region in length (y) on somite number (x) at the mean values of x and y: wild type 1.746 = −0.719 + 0.0983(25.1); heterozygote 1.683 = −0.817 + 0.1037(24.1); homozygote 1.032 = 0.023 + 0.0983(21.5), $P < 0.01$. (C,D) *Alk5*+/− and *Alk5*−/− littermates both with 24 somite pairs (24S) and the respective posterior (hindgut) region after staining for alkaline phosphatase activity. Arrows indicate the most anterior and most posterior PGC, red arrows indicate the gonadal ridge region. The asterisk marks the forelimb bud. (E,F) The total number of PGCs was counted in consecutive sections of E10.5 *Alk5*+/− ($n = 5$) and *Alk5*+/+ ($n = 3$) littermates. Both distribution and total number of PGCs did not differ significantly using the Wilcoxon nonparametric rank test for statistical analysis (medians are depicted by a red stripe). Abbreviations: bw, body wall; gr, gonadal ridges; m, dorsal mesenterium.

Results and discussion

Smad2-dependent signaling is active in PGCs at E10.5 and E12.5

Although TGFβ1 has been described as a chemoattractant for migratory PGCs in vitro, its role in vivo has never been clarified. Between E10.5 and E12.5, PGCs enter the gonadal ridges and are distinguished morphologically from surrounding somatic cells by their typical large round nucleus, but also by the expression of markers for pluripotency, including UTF1 (Okuda et al., 1998) (Figs. 1A, B). At E10.5, not only PGCs but also the surrounding somatic tissue exhibited nuclear staining for active (phosphorylated) TGFβ subfamily R-Smad, PSmad2 (Fig. 1C). At E12.5, both cytoplasmic and nuclear PSmad2 staining was observed in the PGCs (Fig. 1D) as previously described (Chuva de Sousa Lopes et al., 2003). Activation of BMP subfamily R-Smads, PSmad1, 5, and 8 was not observed at E10.5 in PGCs or surrounding somatic tissue (Fig. 1E), but was clearly detected in some PGCs at E12.5, albeit in fewer than had stained for PSmad2 (Fig. 1F). Furthermore, prominent BMP signaling initiated by anti-Müllerian hormone, a member of the BMP subfamily was present in the tissue surrounding the Müllerian duct in males (Fig. 1F), as expected (Jamin et al., 2002). Thus, although the BMP subfamily members apparently have no role in directing PGC migration between E10.5 and E12.5, TGFβ subfamily members may be of importance on the basis of temporal activation of downstream targets via PSmad2.

PGC distribution is abnormal in Alk5-deficient embryos

To investigate whether TGFβs in particular played a role directing migration of PGCs in vivo, the distribution of PGCs was determined in detail in *Alk5*-deficient embryos until E10, when most of the *Alk5* mutants are still viable (Larsson et al., 2001). We observed that the total number of PGCs was similar in wild type and *Alk5*-deficient littermates during early development [3 to 30(S) somites] (Fig. 2A). The loss of this potentially growth-inhibitory pathway did not result in an increase of PGC numbers, suggesting that TGFβs (via ALK5) do not have an anti-mitogenic effect on PGCs in vivo. This contrasts with reports of antimitogenic activity for TGFβ observed in vitro (Godin and Wylie, 1991; Richards et al., 1999).

The distribution of PGCs was similar in wild type and *Alk5*-deficient littermates up to E9 (20S) (data not shown). However, between E9 and E10 (20S–30S), even though the majority of *Alk5*-deficient embryos analyzed were morphologically normal (67% of the *Alk5* mutants versus 83% wild type embryos) and indistinguishable from wild type embryos (Figs. 2C, D), the length of the migratory pathway containing PGCs was significantly shorter in mutant embryos (Fig. 2B) and interestingly the *Alk5*-deficient PGCs were observed to leave the hindgut and reach the

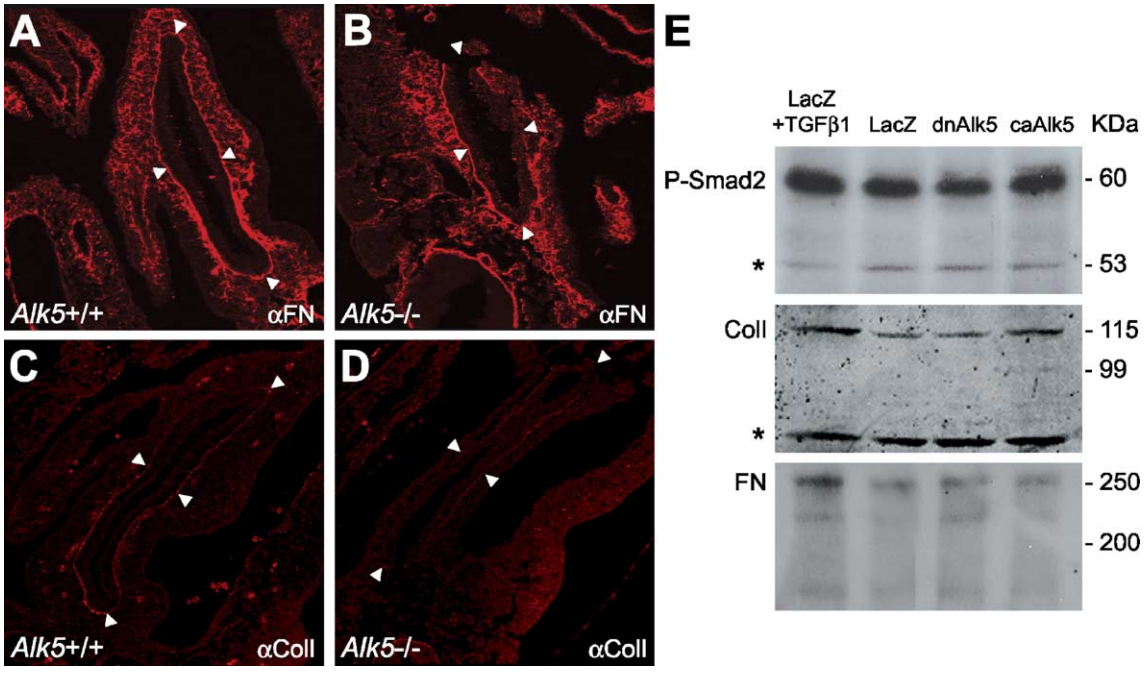

Fig. 3. TGFβ signaling regulates of ECM deposition around the hindgut at E9.5. (A–D) Distribution of fibronectin (FN) and collagen type I (ColI) in E9.5 *Alk5*−/− and wild type littermates analyzed by immunofluorescence on paraffin sections. Arrowheads indicate the boundaries of the gut. (E) Western blot analysis of PSmad2 (P-Smad2), ColI, and FN in dissociated posterior region containing hindgut and dorsal mesenterium of E9.5 embryos, 24 h after infection with either *LacZ*-virus (with or without addition of 10 ng/ml TGFβ1), dn*Alk5*-virus, or ca*Alk5*-virus. *Nonspecific band indicating loading.

gonadal ridges earlier than wild type PGCs (Figs. 2C, D). These data thus suggested that migration of PGCs from the hindgut to the gonadal ridges in vivo occurred more efficiently in the absence of TGFβ signaling and that, surprisingly, TGFβ acted neither as a chemoattractant for PGCs nor controlled the proliferation of the PGCs in vivo until E10.

By E10.5, morphologically normal *Alk5*-deficient embryos could no longer be recovered. We observed that, in E10.5 *Alk5* heterozygotes, neither the total number of PGCs nor their distribution was significantly different from wild type littermate embryos (Figs. 2E, F); thus, until E10.5, only the absence of both *Alk5* alleles affected the migratory behavior of the PGCs.

At E9.5, Alk5 mutants downregulate collagen type I surrounding the gut

In vivo, TGFβ signaling did not regulate PGC migration via a chemotactic mechanism at least until E10, but in contrast seemed to restrict the movement of PGCs out of the hindgut. TGFβ signaling (via ALK5) regulates ECM deposition in part by increasing synthesis of fibronectin and collagen type I (Itoh et al., 2003; Laping et al., 2002). This process has also been proposed as an alternative mechanism regulating the movement of PGCs towards the gonadal ridges. Interestingly, both fibronectin and collagen type I are present along the migratory route of the PGCs (Fujimoto et al., 1985; Soto-Suazo et al., 2004). Therefore, we investigated whether the abnormal distribution of PGCs in *Alk5*-deficient embryos was due to altered deposition of fibronectin or collagen type I. The expression of fibronectin detected by immunofluorescence on paraffin sections of E9.5 embryos was not significantly affected (Figs. 3A, B), whereas expression of collagen type I was downregulated in particular along the gut of E9.5 *Alk5*−/− embryos compared to wild type littermates (Figs. 3C, D). PGCs isolated from E8.5–E12.5 have been shown in vitro to adhere strongly to collagen type IV (Garcia-Castro et al., 1997), suggesting that a reduction in the concentration of collagen specifically around the gut would allow the PGCs to migrate more easily not only through the basal lamina of the hindgut but also from the hindgut to the dorsal mesenterium.

Collagen type I is an early responsive gene to TGFβ Smad-dependent signaling, whereas fibronectin is activated by TGFβ through the MAPK pathway in a Smad-independent fashion (Hocevar et al., 1999; Laping et al., 2002). The observation that the fibronectin levels remain unchanged in *Alk5*-deficient embryos is probably due to the multitude of pathways that lead to the activation of the MAPK signaling consequently regulating fibronectin production.

Laminin and integrin β1, both upregulated by TGFβ signaling (Chakrabarty et al., 2001; Huang and Chakrabarty, 1994; Li et al., 2003; Thibault et al., 2001), have been shown to play a role during the migratory phase of the PGCs from the dorsal mesenterium to the gonadal ridges (reviewed by Wylie and Anderson, 2002). However, lack of integrin β1 (via the isoform β1A, but not β1D) (Anderson et al., 1999; Cachaco et al., 2003) results in defects on migration of PGCs opposite to those observed in embryos lacking ALK5, that is defective colonization of the gonadal ridges; therefore, these molecules are unlikely to be involved in the same pathway controlling PGC migration. Furthermore, impaired TGFβ signaling due to ALK5 deficiency affected PGC migration between E9 and E10, when the PGCs migrate from the hindgut to the dorsal mesenterium, whereas lack of integrin β1 affected migration from the dorsal mesenterium to the gonadal ridges (E10.5–E13.5), but not from the hindgut to the dorsal mesenterium.

In vitro, TGFβ signaling regulates production of collagen type I in hindgut/mesenterium

In the absence of ALK5, collagen type I was downregulated in particular around the hindgut at E9.5. It might be reasonable to suppose that activation of TGFβ signaling would on the other hand result in upregulation of collagen

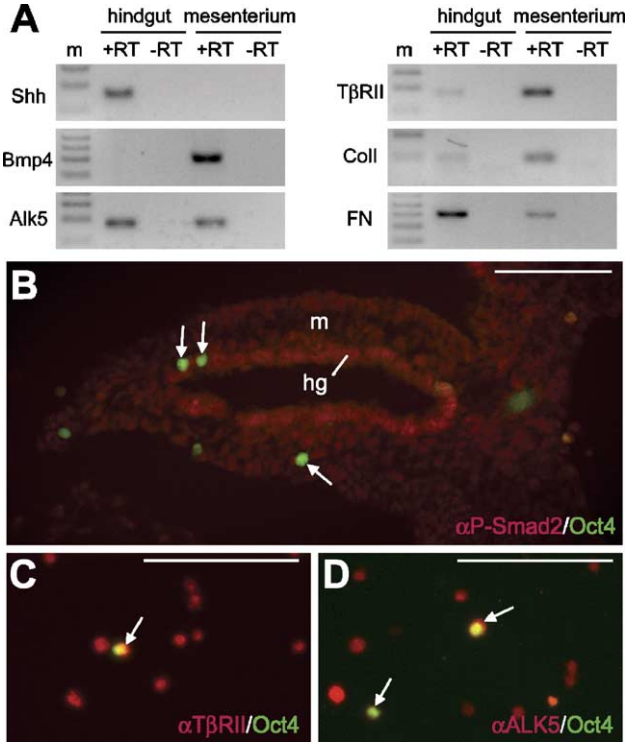

Fig. 4. Analysis of TGFβ signaling in the hindgut and dorsal mesenterium at E9.5. (A) Transcriptional expression of sonic hedgehog (Shh), Bmp4, Alk5, TβRII, collagen type I (ColI), and fibronectin (FN) was analyzed by RT-PCR in isolated hindguts (n = 3) and dorsal mesenteria (n = 5). Contamination by genomic DNA did not occur as indicated by amplification of each sample without reverse transcription (−RT). (m) is the DNA ladder. (B) Transverse cryosection of the posterior region of Oct4ΔPE:*gfp* E9.5 embryo immunostained for PSmad2. Abbreviations: da, dorsal aorta; hg, hindgut; m, mesenterium. (C,D) Immunostaining for TβRII or ALK5 and Oct4 in dispersed PGCs (white arrows) and surrounding somatic cells at E9.5. Scale bar: 100 μm.

type I. To determine whether this was the case, the posterior region of E9.5 embryos consisting of hindgut and dorsal mesenterium was dissociated, plated, and infected with either constitutively active (ca)*Alk5*, dominant-negative (dn)*Alk5*, or *LacZ* expressing adenovirus (Fujii et al., 1999). Infection conditions were optimized using adenovirus expressing *LacZ* (results not shown). 24 h after infection, the cells were used for Western blotting. The results indicated that the levels of PSmad2 were high, even in the presence of dnALK5. This high basal level may be the result of activation by other members of the TGFβ subfamily (including nodal and activin), that signal via other type I receptors than ALK5, but still induce phosphorylation of Smad2. Nonetheless, cells either infected with *LacZ*-virus and treated with 10 ng/ml TGFβ1 1 or infected with ca*Alk5*-virus showed a relative increase in the levels of both PSmad2 and collagen type I (Fig. 3E). Together, this clearly demonstrated that the hindgut/mesenterium region responds to changes in TGFβ signaling both in vivo and in vitro by regulating the expression of collagen type I.

The levels of fibronectin were not significantly affected by expression caALK5, but were increased by treatment with TGFβ1 (Fig. 3E). Although caALK5 has been shown to induce fibronectin expression (M.J. Goumans, personal communication), the lack of upregulation by caALK5 can be explained by the fact that caALK5 is only expressed approximately 12 h after infection. Although this is sufficient to increase the production of collagen type I (an early TGFβ1 target gene), it does not increase the expression of fibronectin (upregulated approximately 16 h after receptor activation) (Laping et al., 2002).

Smad2-dependent signaling is active in the hindgut at E9.5

To understand more about the mechanism by which collagen type I deposition occurs at E9.5, we investigated whether collagen type I was produced either by the hindgut or by the surrounding dorsal mesenterium cells. Therefore, RT-PCR was performed on isolated hindguts and separately on dorsal mesenterium cells. As expected, hindgut cells expressed sonic hedgehog (Shh), but not Bmp4, whereas dorsal mesenterium cells expressed Bmp4, but not Shh (Fig. 4A) (Wells and Melton, 1999). Both tissues expressed Alk5 and TβRII, indicating ability to respond to TGFβs (Fig. 4A). Additionally, both tissues produced collagen type I and fibronectin (Fig. 4A). Next, we studied if TGFβ signaling was indeed active in both tissues by looking at the expression of PSmad2 in cryosections of the posterior region of Oct4ΔPE:*gfp* transgenic embryos, in which the PGCs are positive for GFP (Yeom et al., 1996). Prominent nuclear PSmad2 was previously described by us in the gut at E8.5 (Chuva de Sousa Lopes et al., 2003). At E9.5, we observed high levels of PSmad2 in the hindgut, but not in

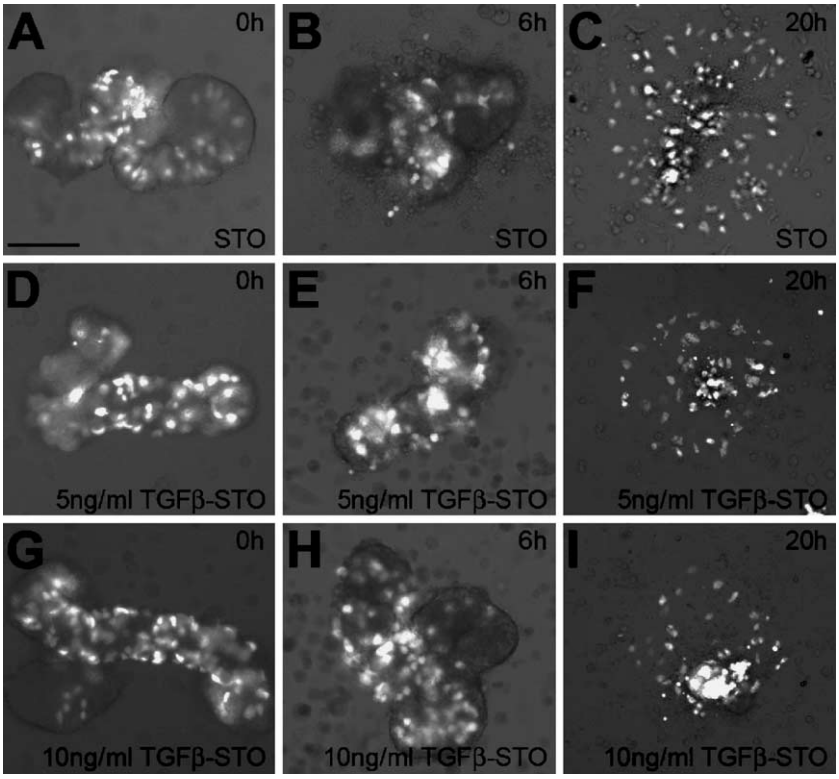

Fig. 5. TGFβ1 inhibits the movement of PGCs out of the hindgut at E9.5. (A–I) Oct4ΔPE:*gfp* E9.5 hindguts were isolated free from dorsal mesenterium cells (A,D,G) and cultured for 20 h on STO feeder cells in the absence (A–C) or presence of 5 ng/ml (D–F) and 10 ng/ml (G–I) exogenous TGFβ1. Hindguts were monitored 6 h after isolation (B,E,H). Scale bar: 100 μm.

the dorsal mesenterium (Fig. 4B), suggesting that in vivo (TGFβ) Smad2-dependent signaling may regulate collagen type I in the hindgut. In addition, PGCs showed low levels of PSmad2 (Fig. 4B), but PGCs dispersed from the posterior region of E9.5 embryos express both TβRII and ALK5 and are therefore able to respond to TGFβs (Figs. 4C, D).

In vitro, TGFβ1 and collagen type I restrict the movement of PGCs out of the hindgut

If indeed TGFβ signaling acted on the hindgut cells thereby regulating the movement of PGCs out of the hindgut, we would expect that addition of exogenous TGFβ1 to explants of hindgut would result in restricted migration of PGCs. To study this, we isolated E9.5 Oct4ΔPE:*gfp* whole hindguts free of mesenterium cells and cultured them on STO feeder cells for a period of 20 h. PGCs were shown to migrate directly on STO feeder cells (Ffrench-Constant et al., 1991). Although we cannot exclude a direct effect on the STO cells, increasing concentrations of TGFβ1 significantly reduced the mobility of PGCs from the hindgut into the STO feeder layer (Fig. 5), in agreement with the idea that TGFβ signaling would restrict PGC migration out of the hindgut in vivo.

Finally, to learn more about the direct role of collagen type I in the migration of PGCs out of the hindgut, hindguts from E9.5 Oct4ΔPE:*gfp* embryos isolated free of mesenterium cells were also cultured for 20 h on increasing concentrations of collagen type I. Independent of the concentration of collagen type I used, PGCs adhered to the substrate and migrated as well as hindgut cells, being observed in the boundary of the explant (Fig. 6). However, the higher the concentration of collagen type I, the less PGCs migrated, remaining clustered together at the center of the explant, supposedly where it attached to the substrate. This indicated that PGCs adhere strongly to collagen type I. From our results, it is evident that both collagen type I and TGFβ1 (probably by direct induction of collagen type I) affected the ability of the PGCs to leave the hindgut and to be migratory in culture.

In summary, we have clarified the role of TGFβ signaling pathway via ALK5 during the migration of PGCs in vivo and demonstrate for the first time that TGFβ signaling plays no role regulating the proliferation of PGCs or acting as a chemoattractant until E10. However, by regulating collagen type I deposition around the hindgut, TGFβ signaling indirectly restricts the migration of PGCs from the hindgut to the dorsal mesenterium.

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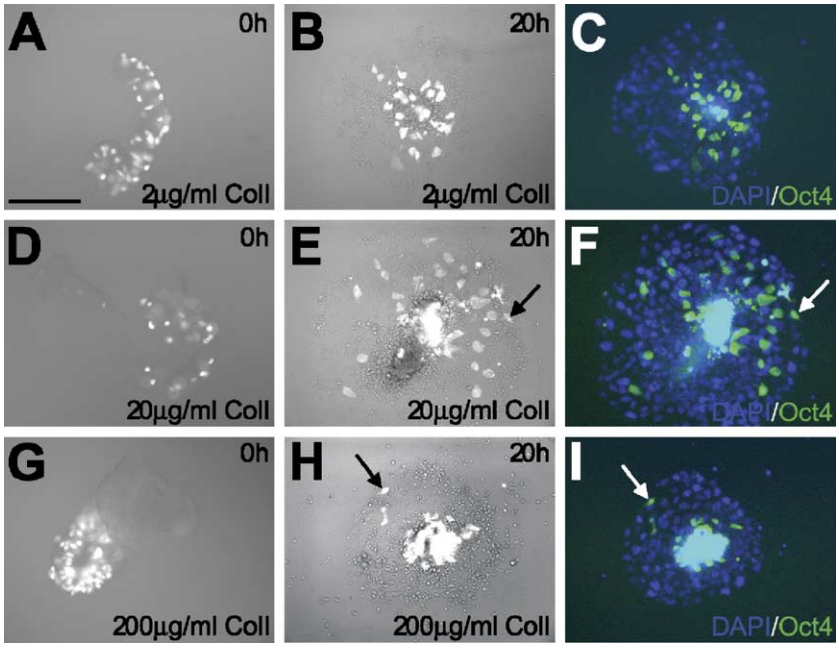

Fig. 6. Collagen type I restricts the movement of PGCs out of the hindgut at E9.5. (A–I) Oct4ΔPE:*gfp* E9.5 hindguts were isolated free of dorsal mesenterium cells (A,D,G) and cultured for 20 h on coverslips coated with 3 different concentrations of collagen type I, namely, 2 μg/ml (A–C), 20 μg/ml (D–F), and 200 μg/ml (G–I). Panels C, F, and I represent the same explant as in panels B, E, and H, respectively, but showing in addition to the GFP (Oct4) staining the PGCs, DAPI staining showing all nuclei to clearly visualize the boundaries of the explants. Arrows represent PGCs at the boundary of the explant. Scale bar in panels A, D, and G represents 200 μm, and in panels B, C, E, F, H, and I, scale bar represents 100 μm.

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